Efek Penghambatan Minyak Buah Merah Terhadap Melanogenesis Melalui Degradasi Tirosinase

Inhibitory Effect of Buah Merah Oil on Melanogenesis via Degradation of Tyrosinase

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ABSTRAK: Buah merah (*Pandanusconoideus*) terdistribusi secara eksklusif di pulau Papua dan daerah sekitarnya. Ekstrak minyak dari buah merah kaya dengan kandungan lipid, karotenoid dan vitamin E. Kami telah menguji efek minyak buah merah terhadap melanogenesis pada sel melanoma B16. Dengan keberadaan a*lpha- Melanocyte Stimulating Hormone* (α-MSH), sel melanoma akan terstimulasi meningkatkan sintesis melanin. Minyak buah merah menghambat sintesis melanin yang diinduksi oleh α-MSH tanpa adanya efek sitotoksisitas. Penurunan melanogenesis berhubungan dengan menurunnya aktivitas enzim tirosinase dan menurunnya tingkat ekspresi protein tirosinase. Tirosinase adalah glikoprotein membrane tipe 1 yang merupakan enzim penting yang terlibat dalam sintesis melanin. Selanjutnya, minyak buah merah menurunkan kadar melanin dengan cara menurunkan jumlah dan aktivitas enzim tirosinase, dimana tingkat mRNA tirosinase tidak berubah. Perlakuan dengan inhibitor proteosoma MG132 diblokir sehingga menurunkan pengaturan melanogenesis oleh minyak buah merah. Analisis immunopresipitasi juga menunjukkan bahwa perlakuan dengan minyak buah merah memodulasi ubiquitinasi tirosinase sehingga minyak buah merah dapat meningkatkan jumlah tirosinase terubiquitinasi. Hasil secara keseluruhan menunjukkan bahwa efek depigmentasi minyak buah merah berhubungan dengan penghambatan ekspresi tirosinase melalui degradasi tirokinase yang dimediasi ubiquitin proteasome.

Kata kunci: buah merah, melanogenesis, tirosinase, degradasi, ubiquitin-proteasome

ABSTRACT: Buah Merah (*Pandanusconoideus*) is exclusively distributed in Papua island and its neighboring areas. The extract oil from fruits (Buah Merah oil) contains rich in lipids, carotenoids and vitamin E. We assessed the effects of Buah Merah oil on melanogenesis in B16 melanoma cells. In the presence of alpha-melanocyte stimulating hormone (α-MSH), B16 melanoma cells are stimulated to enhance melanin synthesis. Buah Merah oil inhibited α-MSH-induced melanin synthesis with no cytotoxicity. This decrease in melanogenesis was correlated with reduced enzyme activity and decreased protein expression levels of tyrosinase. Tyrosinase is a type 1 membrane glycoprotein that is the critical rate-limiting enzyme involved in melanin synthesis. Furthermore, the Buah Merah oil-induced decrease in melanin content was accompanied by a decrease in the amount and activity of tyrosinase whereas the mRNA level of tyrosinase was unchanged. Moreover, treatment with proteasome inhibitor MG132 blocked the down-regulation of melanogenesis by Buah Merah oil. Immunoprecipitation analysis also revealed that treatment with Buah Merah oil modulated the ubiquitination of tyrosinase, that is Buah Merah oil increased the amount of ubiquitinatedtyrosinase. Taken together, the present results indicate that the depigmenting effect of Buah Merah oil might be due to inhibition of tyrosinase expression through the ubiquitin proteasome-mediated degradation of tyrosinase.

Keywords: buah merah, melanogenesis, tyrosinase, degradation, ubiquitin-proteasome

1. Introduction

Melanin, which is the pigment of skin color, is synthesized in specialized organelles, termed melanosomes. Melanosomes enclosing melanin polymer are transferred from melanocyte to surrounding keratinocytes in human epidermis. The quantity, type, and distribution of melanin in keratinocytes are one determinant of human skin color (Parvez *et al.*, 2006; Wakamatsu *et al.,* 2006). The main physiological stimulus of melanogenesis is the ultraviolet radiation of solar light, which can act directly on melanocytes or indirectly induce the release of keratinocyte-derived factors such as α-MSH (α-melanocyte stimulating hormone) (Hunt *et al.*, 1994), endothelin (ET)-1 (Imokawa *et al.,* 1997) or PGE2 (Scott *et al.,* 2004). Melanin pigmentation in the skin is the major defense reaction against ultraviolet radiation. However, abnormal pigmentation such as blotches, freckles, and other forms of melanin hyperpigmentation can cause serious aesthetic problems (Briganti, Camera & Picardo, 2003).

In melanosomes, melanogenesis is carried out by means of a specific enzymatic pathway initiated by tyrosinase. Tyrosinase (EC 1.14.18.1) is a type 1 membrane glycoprotein that is the critical rate-limiting enzyme involved in melanin synthesis (Hearing & Jiménez, 1987; Marmol V, Beermann F (1996). It catalyzes two distinct reactions of melanin synthesis, the hydroxylation of L-tyrosine by monophenolase and the oxidation of L-dopa to dopaquinone by diphenolase (Hearing & Tsukamoto, 1991). α-MSH binds to its specific receptor (MC1R) and increases cAMP. cAMP activates melanogenesis by activating microphthalmia-associated transcription factor (MITF), a melanocyte-specific transcription factor (Buscà & Ballotti, 2000). MITF regulates the expressions of tyrosinase, tyrosinase-related protein 1 (TRP1), and tyrosinase-related protein 2 (TRP2). Tyrosinase is initially synthesized in the endoplasmic reticulum (ER) and is matured by complex sugar modifications in the Golgi apparatus. Following processing in the Golgi apparatus, tyrosinase traffics through the trans-Golgi network to melanosomes (Orlow, 1995) and then melanin is synthesized. Importantly, tyrosinasecatalyses the rate-limiting reaction of the melanogenic process, and thus, melanin production is mainly controlled by the expression and activity of tyrosinase.

Pandanusconoideus is naturally and exclusively grown in Papua island and its neighboring areas. It is called Buah Merah, meaning red fruits, by native people. Buah Merah is one of varieties of pandanaceae. The extract oil from fruits (Buah Merah oil) contains rich in lipids, carotenoids and vitamin E. Carotenoids of Buah Merah oil comprise mainly alpha- and beta-carotene as well as alpha- and beta-cryptoxanthin (Nishigaki *et al.,* 2011; Wada *et al.,* 2013).

In this study, we investigated whether Buah Merah oil exhibits inhibitory effect against α-MSH-induced melanogenesis using B16 melanoma cells.

2. Method

2.1. Reagent

Buah Merah oil was provided by M&K Laboratories Inc. (Nagano, Japan). α-MSH was purchased from PEPTIDE INSTITUTE, INC. (Osaka, Japan). $_{L}$-3,4-dihydroxyphenylalanine ($_{L}$-dopa) was obtained from Sigma-Aldrich (St Louis, MO, USA).Dimethyl sulfoxide(DMSO) and cycloheximide were purchased from NacalaiTesque Inc.(Kyoto, Japan). MG132 was obtained from Abcam Inc. (Cambridge, MA, USA).

2.2. Cell culture

B16 melanoma cells (RCB1283) were obtained from Riken Cell Bank (Ibaraki, Japan). This cell line is derived from C57BL/6 mice. B16 melanoma cells were cultured in Dulbecco's modified Eagles medium (DMEM) with 10% fetal bovine serum and penicillin/streptomycin in air containing 5% CO_2 at 37°C.

2.3. Cell viability

The cell proliferation assay was carried out using 3- (4, 5-dimethylthiazol-2-yl) -2, 5-diphenyltetrazolium bromide (MTT). B16 melanoma cells were cultured at 4×10^3 cells/well with Buah Merah oil for 96 h in 96-well plates. After 96 h, 10 μL of MTT solution (5 mg/mL in PBS) was added. After incubation at 37°C for 3 h, the medium was gently removed and 100 μL of DMSO was added. The absorbance of each well was measured at 570 nm using a spectrophotometer (Sunrise Rainbow, Tecan Japan Co., Ltd., Kanagawa, Japan).

2.4. Measurement of melanin content

B16 melanoma cells were cultured at 1×10^5 cells/dish with 10 nM α-MSH and various concentrations of Buah Merah oil for 96 h in 6 cm dishes. After washing with PBS, the cells were harvested by trypsinization. The cell pellets were solubilized in 150 μL of 1 N NaOH at 100°C for 10 min. The absorbance was measured at 405 nm using a spectrophotometer.

2.5. Tyrosinase activity assay

B16 melanoma cells were cultured at 1×10^5 cells/dish with 10 nM α-MSH and various

concentrations of Buah Merah oil for 96 h in 6 cm dishes. The cells were lysed by incubating at 4°C for 30 min in RIPA buffer (150 mMNaCl, 1% NP-40, 0.5% sodium deoxycholate, 50 mMTris-HCl, pH 7.6 containing 0.1% SDS). The lysates were centrifuged at 12,000×g for 10 min to obtain the supernatant. The reaction was carried out by incubating the supernatant with 50 mM phosphate buffer, pH 6.8 and 10 mM L-dopa. After incubation at 37°C for 20 min, dopachrome formation was monitored by measuring absorbance at wavelength 475 nm.

2.6. Antibodies

This research was performed with the following commercially available antibodies: anti-goat tyrosinase (C-19), anti-rabbit tyrosinase (H-109), anti-rabbit MITF (H-50), anti-mouse Ub (P4D1) from Santa Cruz BiotechnologyInc. (Santa Cruz, CA, USA). Anti-mouse β-actin was obtained from Sigma-Aldrich (St Louis, MO, USA).

2.7. Western Blot Analysis

To measure tyrosinase content, cells were treated withBuah Merah oil for 96 h. For evaluating MITF content, cells were treated withBuah Merah oil for 72 h. To measure tyrosinase degradation, cells were pretreated with Buah Merah oil for 72 h and then treated with cycloheximide (1 μg/mL) for an additional 4 or 8 h. Cells were treated with Buah Merah oil, with or without incubation in the presence of proteasome inhibitors (MG132 at 120 nM) for 72 h.

The cells were lysed by incubating at 4°C for 30 min in RIPA buffer. After incubation, the lysates were centrifuged at 12,000×g for 10 min and supernatants were isolated. Proteins were extracted by boiling the tissues in 0.5 mMTris/HCl, pH 6.8, glycerol, 10% SDS, 0.1% bromophenol blue, and 2-mercaptoethanol. The proteins were electrophoresed by using 12.5 or 15% SDS-PAGE gel at 100 V for 2 h. After fractionating, the proteins were transferred onto a poly (vinylidenedifluoride) (PVDF) membrane at 100 mA for 1h. The membranes were incubated with the primary antibodies and further incubated with horseradish peroxidase-conjugated secondary antibodies. Detection was achieved by using an ECL kit (NacalaiTesque Inc., Kyoto, Japan). The density of the bands was measured by using NIH Image.

2.8. Real-time quantitative PCR analysis

B16 melanoma cells were cultured at 1×10^5 cells/dish with 10 nM α-MSH and various Buah Merah oil for 96h in 6cm dishes. After 72 h, total RNA was isolated from each treatment group using Sepasol-RNA I Super G (NacalaiTesque Inc., Kyoto, Japan). From each sample, total RNA was reverse transcribed to cDNA using the ReverTra Ace qPCR RT Kit (Toyobo, Osaka, Japan). The reverse transcriptional products containing 1.0 μg of cDNA were amplified in a reaction mixture (20 μL) containing 10 μL Thunderbird™ SYBR qPCR Mix and 6 pmol of each forward and reverse primers by ABI Prism 7000 Real Time PCR System (Applied Biosystems, Foster City, CA, USA). Real-time quantitative PCR analysis was carried out under the following condition: 40 cycles of denaturation at 95°C for 15 s, annealing at 60°C for 1 min. Relative levels of tyrosinase mRNA was expressed compared to β-actin mRNA. Primers used were for tyrosinase: forward primer 5'-CAAGTACAGGGATCGGCCAAC-3' and reverse primer 5'-GGTGCATTGGCTTCTGGGTAA-3', for β-actin: forward primer 5'-AGCCTTCCTTCTTGGGTATG-3' and reverse primer 5'-CAGGAGGAGCAATGATCTTG-3' (Invitrogen, Life Technologies, Carlsbad, CA,USA).

2.9. Immunoprecipitation

Cells were treated with Buah Merah oil in the presence of proteasome inhibitors MG132 for 72h. Cell extracts (200 μg of total protein in 400 μL of RIPA buffer) were incubated with 20 μL of Protein A/G PLUS-Agarose with continuous mixing for 1 h at 4°C. After centrifugation (2,000×*g* for 1 min at 4°C), the supernatants were used as pre-cleared cell extracts. The pre-cleared cell extracts were incubated with 4μL of anti-rabbit tyrosinase (H-109). After continuous mixing for 1 h at 4°C, 20 μL of Protein A/G PLUS-Agarose suspended in RIPA buffer was added and mixed further for 1 h at 4°C. After the antigen-antibody complexes were precipitated by brief centrifugation, the pellets were washed RIPA buffer. Finally, absorbed proteins were eluted with 20 μL of Tris-glycine SDS sample buffer with 2-mercaptoethanol at 95°C for 5 min. Each supernatant was separated on 12.5% Tris-glycine SDS gels and then transferred to PVDF membranes. Ub (P4D1) was used to detect ubiquitinatedtyrosinase. The detection method was the same as the one indicated in the protocol for Western blot analysis.

2.10.Statistical analysis

Statistical significance was tested with Student t-test, Dunnett's test or Turkey test. All results are presented as the mean ± S.E. Differences of $P< 0.05$ were considered significant.

3. Result and discussion

3.1. *Effect of buah merah oil on melanin content*

When B16 melanoma cells were incubated with α-MSH, the pellets turned black, indicating increased melanin content. Buah Merah oil

decreased melanin content in the presence of α-MSH without cytotoxicity (Fig.1a, b).

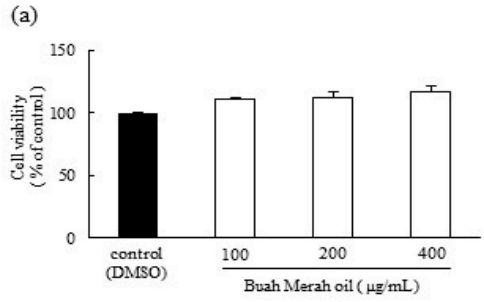

Figure 1.a Cell viability test of Buah merah oil

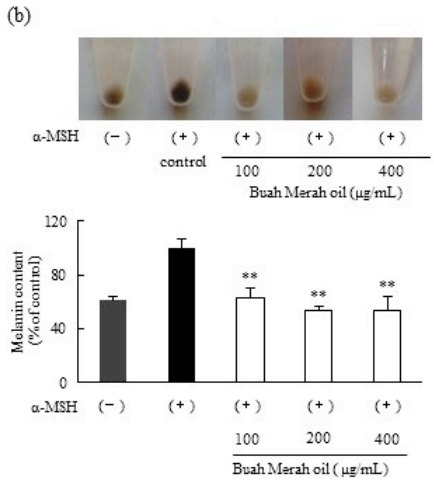

Figure 1.b Melanin content test of Buah Merah

Although melanin plays a critical role to protect skin from the harmful effects of ultraviolet radiation of solar light, excess melanin synthesis can lead to hyper-pigmentation disorders (Briganti, Camera, & Picardo, 2003). Therefore, development of melanin synthesis inhibitors is of great important. Mechanisms of agents causing hypopigmentation have been reviewed (i) tyrosinase inhibition, maturation and enhancement of its degradation; (ii) MITF inhibition; (iii) downregulation of MC1R activity; (iv) interference with melanosome maturation and transfer; (v) melanocyte loss, desquamation and chemical peeling (Solano, Briganti, Picardo, Ghanem, 2006). Tyrosinase inhibition is the most common approach to achieve skin hypopigmentation (Ando, 2007).

3.2. *Effect of buah merah oil on tyrosinase activity*

Buah Merah oil decreased cellular tyrosinase activity (Fig.1c) and expression of tyrosinase protein (Fig.1d).

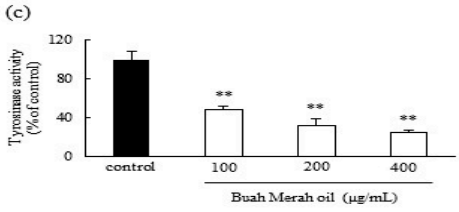

Figure 1.c Cellular tyrosinase activity test of Buah Merah oil

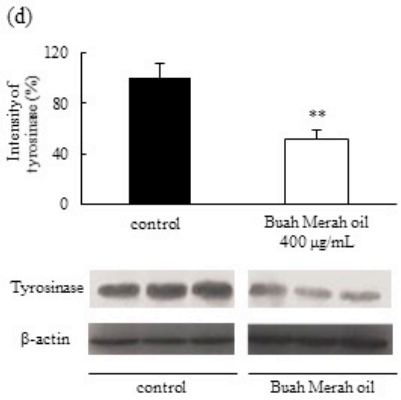

Figure 1.d Expression of Tyrosinase protein test of Buah merah oil

3.3. *Effects of buah merah oil on the expression of tyrosinase synthesis*

We investigated to determine whether Buah Merah oil affects MITF protein and tyrosinase mRNA expression. MITF protein and tyrosinase mRNA were not altered by Buah Merah oil (Fig .2a,b). These results indicate that the decrease in tyrosinase protein levels by Buah Merah oil in B16 melanoma cells is not due to the reduction of tyrosinase mRNA levels.

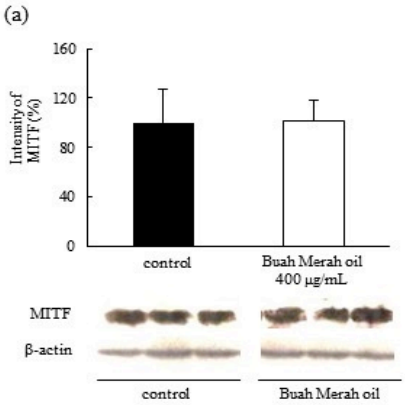

Figure 2.a MITF protein test of Buah merah oil

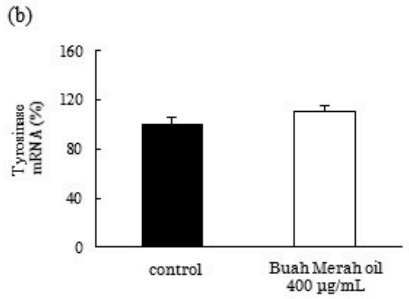

Figure 2.b Tyrosinase mRNA expression test of buah merah oil

However, Buah Merah oil did not decrease tyrosinase mRNA level and MITF protein level in B16 melanoma cells (Fig.2). These results indicate that Buah Merah oil does not affect transcription of tyrosinase. Proteolysis can be considered as another mechanism of reducedtyrosinase protein level. Tyrosinase is degraded by the proteasomal and the lysosomal pathway. It has been reported that phospholipase D2 (Kageyama, 2004), linoleic acid (Ando *et al*., 2004; Ando *et al*., 2006) and, and terrain (Park, 2009) enhance tyrosinase degradation via ubiquitin-proteasome pathway and reduce melanogenesis. Recently, Bellei et al. stated that p38 regulates melanogenesis via promotion of proteasomal degradation of tyrosinase (Bellei, 2010). In 2004, Hall *et al.*, reported that 25-hydroxycholesterol decreases melanin synthesis and accelerates degradation of glycosylated tyrosinase after the ER and the Golgi apparatus modification of tyrosinase via a proteasome-independent mechanism (Hall & Krishnamoorthy, Orlow, 2004). Hall *et al*., stated that phenylthiourea promotes tyrosinase degradation following maturation in the Golgi apparatus in an endosomal/lysosomal cysteine proteasome-dependent manner in cultured melanocyte (Hall, Orlow, 2005). Fujita *et al.* reported that inulavosin, a melanogenesis inhibitor, accelerates tyrosinase degradation by mistargeting of tyrosinase to lysosomes (Fujita, 2009). To investigate whether Buah Merah oil affects proteolysis of tyrosinase, Western blot analysis of tyrosinase was performed in B16 melanoma cells treated with cycloheximide, a protein synthesis inhibitor, after treatment with Buah Merah oil.

3.4. *Acceleratory effect of buah merah oil on tyrosinase degradation*

We assessed the rate of proteolysis of tyrosinase using cycloheximide, a protein synthesis inhibitor, after treatment with Buah Merah oil. When measured as relative intensity of the tyrosinase band at 4 or 8 h compared to the initial intensity, the rate of tyrosinase protein degradation in Buah Merah oil treated cells was significantly higher than that of untreated (DMSO) cells (Fig.3a). This result indicates that the degradation of tyrosinase was accelerated by treatment with Buah Merah oil. The reduced tyrosinase protein that occurred in response to Buah Merah oil treatment was related to proteasomal degradation. Therefore, we treated B16 melanoma cells with low concentrations of proteasome inhibitor MG132 for 72 h in the presence of Buah Merah oil. The decrease of tyrosinase induced by Buah.

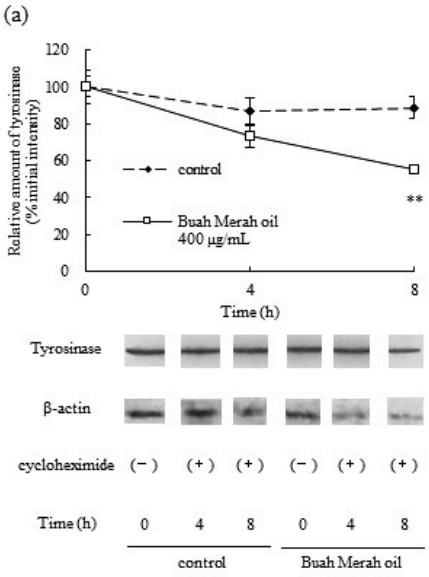

Figure 3.a Proteolysis of tyrosinase test of Buah merah oil

We next examined whether the Buah Merah oil - induced decrease of tyrosinase was due to its proteolytic degradation by proteasomes. Buah Merah oil decreased the level of tyrosinase. The decrease of tyrosinase induced by Buah Merah oil could be blocked by MG132,proteasome inhibitor, after co-incubation for 72 h (Fig.3b).

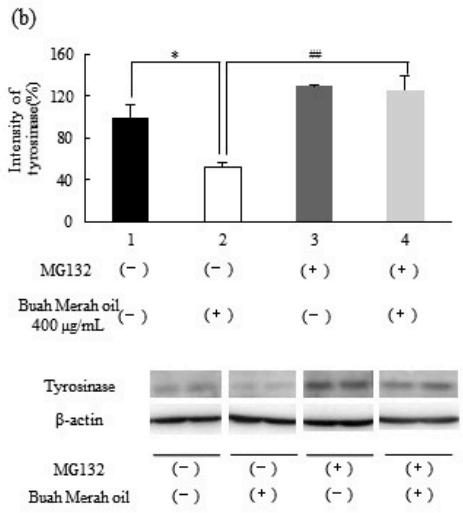

Figure 3.b Tyrosinase test of buah merah oil

To elucidate the mechanism by which the ubiquitin-proteasome pathway is involved in Buah Merah oil induced tyrosinase degradation, we performed immunoprecipitation analysis to evaluate the effect of Buah Merah oil on the ubiquitination of tyrosinase. To do that, it was necessary to stabilize ubiquitinated proteins, which would normally be immediately degraded by proteasomes, and we used MG132 at 120 nM to accomplish this. Interestingly, treatment with Buah Merah oil in the presence of MG132 showed increase in ubiquitinatedtyrosinase compared with the MG132-treated control (Fig.3c).

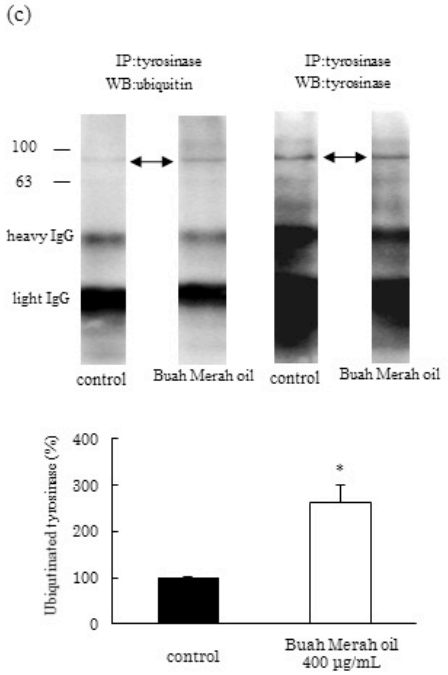

Figure 3.c Effect of Buah Merah oil on the ubiquitination of tyrosinase

These findings demonstrate that Buah Merah oil decreases the tyrosinase via ubiquitin dependent proteasomal degradation. This depigmenting mechanism is different from many skin-whitening agents such as kojic acid (Mishima, Hatta, Ohyama & Inazu, 1988) and arbutin (Maeda & Fukuda, 1996). Further study will be needed to clarify the precise mechanisms that Buah Merah oil regulates the ubiquitination of tyrosinase which then become targeted for proteasomal degradation. Despite many tyrosinase inhibitors in vitro, few agents are able to induce effects in clinical trials.

4. Conclusion

This study clearly indicates that Buah Merah oil inhibits tyrosinase protein expression, which results in downregulation of melanin production. On the basis of these results, we examined the effects of Buah Merah oil on tyrosinase mRNA expression and MITF, which is a transcription factor that effectively transactivates the expression of tyrosinase and its related genes by binding to their common promoters.

Buah Merah oil induces downregulation of melanin production in α-MSH-stimulated B16 melanoma cells through the ubiquitin proteasome-mediated degradation of tyrosinase.

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